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Oxidative Stimulation of Glutathione Synthesis in *Arabidopsis thaliana* Suspension Cultures[1]

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A system based on *Arabidopsis thaliana* suspension cultures was established for the analysis of glutathione (GSH) synthesis in the presence of hydrogen peroxide. Mild oxidative stress was induced by use of the catalase inhibitor, aminotriazole, and its development was monitored by measurement of the oxidative inactivation of aconitase. Addition of 2 mM aminotriazole resulted in a 25% decrease in activity of aconitase over 4 h. During the subsequent 10 h, no further decrease in aconitase activity was measured despite a sustained inhibition of catalase. In combination with our failure to detect significant increases in the level of lipid peroxidation, another marker indicative of oxidative injury, these data suggest that although hydrogen peroxide initially leaked into the cytosol, its accumulation was limited by a cytosolic catalase-independent mechanism. A 4-fold increase in the level of GSH, which was almost exclusively in the reduced form, was observed under the same treatment. To determine to what extent this increase in reduced GSH played a role in limiting the accumulation of hydrogen peroxide in the cytosol, we inhibited GSH synthesis with buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase. No significant oxidative injury was detected as a result of treatment with 50 μM BSO alone, and furthermore, this treatment had no effect on cell viability. However, addition of 2 mM aminotriazole to cells preincubated with 50 μM BSO for 15 h led to a rapid loss of aconitase activity (75% in 4 h), and significant accumulation of products of lipid peroxidation. Within 72 h, cell viability was lost completely. After removal of BSO from the growth medium, GSH levels recovered to normal over a period of 20 h. Addition of 2 mM aminotriazole to cells at different time points during this recovery period demonstrated a strong correlation between the level of reduced GSH and the degree of protection against oxidative injury. These data strongly suggest that the induction of GSH synthesis by an oxidative stimulus plays a crucial role in determining the susceptibility of cells to oxidative stress.

GSH is an important antioxidant in all aerobic organisms and plays a crucial role in the defense against activated oxygen species arising as by-products of metabolism (Meister, 1983; Alscher, 1989; Sen Gupta et al., 1991). In plants, the potential for the production of activated oxygen species is greatly enhanced by a wide range of environmental stresses, and it is thought that the ensuing damage results from the accumulation of these species to levels exceeding the antioxidant capacities of the cell (Hamilton, 1991). A potentially decisive factor in determining the outcome of an oxidative stress is the speed with which plants can activate their antioxidant reserves (Lee and Bennet, 1982; Guri, 1983; Madamanchi and Alscher, 1991). Correlative studies have indicated that this response is an important aspect of stress tolerance.

GSH is likely to play an important role in stress tolerance and has been implicated in the adaptation of plants to environmental stresses such as drought (Dhindsa and Matowe, 1981), atmospheric pollution (Lee and Bennet, 1982; Guri, 1983; Sen Gupta et al., 1991), and extremes of temperature; furthermore, its synthesis is induced in response to these stresses (Alscher, 1989). However, as yet, no studies have demonstrated an absolute dependence on GSH for stress tolerance, nor have studies demonstrating an induction of GSH synthesis in response to an oxidative stimulus been extended to demonstrate that this elevation has any physiological significance. Studies in animal systems have shown that depletion of GSH renders cells susceptible to oxidative cytolysis (Arrick et al., 1982); yet despite their potential relevance, no such analyses have been carried out in plants.

A major impediment to progress in understanding the metabolic and molecular events regulating the synthesis of GSH has been the problems encountered in the purification and characterization of the relevant biosynthetic enzymes. However, recent progress has established that in plants, as in all other organisms studied to date, GSH is synthesized by a two-step process. The first step, catalyzed by γ-glutamylcysteine synthetase (Hell and Bergmann, 1990), results in the production of γ-glutamylcysteine; the second, catalyzed by GSH synthetase, produces GSH through the addition of Gly (Rennenberg, 1982; Alscher, 1989).

GSH synthesis has been shown to respond either directly or indirectly to hydrogen peroxide (Smith et al., 1984; Smith, 1985). The primary aim of this work was to develop a system in which hydrogen peroxide could be synthesized intracellularly in a controlled manner and GSH synthesis could be measured in parallel. By virtue of their undifferentiated composition and ease of manipulation, suspension cultures offer the simplest model system of choice. The plant species chosen was *Arabidopsis thaliana* because it provides a system in which biochemical parameters can be defined alongside gene cloning and genetic analyses. The studies of Smith and colleagues were fundamental in drawing attention to the link between hydrogen peroxide and GSH synthesis (Smith et al., 1984, 1985; Smith, 1985). They showed that inhibition of catalase by aminotriazole leads to leakage of hydrogen per-

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Abbreviation: BSO, L-buthionine-[*S*,*R*]-sulfoximine.

oxide from the peroxisomes and to a stimulation of GSH synthesis. We chose to adopt a similar approach for the generation of hydrogen peroxide intracellularly in *Arabidopsis* suspension cultures.

A second important question we wished to address was how to measure the presence and accumulation of hydrogen peroxide. Oxidative stress results from an accumulation of reactive oxygen species above the antioxidant capacities of the cell. Since the signals regulating GSH synthesis are probably generated early in this response, we sought to identify suitable biochemical markers that could reveal the presence of low levels of hydrogen peroxide. Current methods for determination of hydrogen peroxide generated intracellularly are either very complex or too insensitive. It has been demonstrated recently in vitro that the Krebs-cycle enzyme aconitase (EC 4.2.1.3) undergoes a rapid inactivation in the presence of hydrogen peroxide (Verniquet et al., 1991). This inactivation displays all the characteristics necessary for its use as a suitable specific marker of low levels of hydrogen peroxide in vivo. Plant extracts contain appreciable aconitase activity that is rapidly and irreversibly inhibited by low levels of hydrogen peroxide (Verniquet et al., 1991). In principle, the degree of inhibition should give an indication of the level of hydrogen peroxide present and could also give a measure of the antioxidant capacities of the cell.

In this paper we describe the use of heterotrophic *Arabidopsis* cell-suspension cultures to investigate the regulation of GSH levels in response to the presence of low levels of hydrogen peroxide generated by aminotriazole-induced inhibition of catalase. Changes in aconitase activity in vivo were used to monitor the onset and development of hydrogen peroxide accumulation. In addition, by blocking GSH synthesis using the selective inhibitor of γ-glutamylcysteine synthetase BSO (Griffith and Meister, 1979), we analyzed the physiological significance of changes in the levels of GSH for metabolic regulation and cellular homeostasis in cultures subjected to mild oxidative stress.

MATERIALS AND METHODS

Cell Culture

Seeds of *Arabidopsis thaliana* var Erecta (provided by Dr. Bernard Mulligan, Nottingham, UK) were surface sterilized in 10% (v/v) sodium hypochlorite, rinsed, and plated on solid Gamborg B5 medium (Sigma) containing 0.8% (w/v) agar. Germination and growth were in a controlled environment chamber: 24°C, 70% humidity, and 16-h photoperiod. Callus formation was induced by placing stem explants onto callus induction medium (Gamborg B5, Glc 2% [w/v], agar 0.8% [w/v], Mes 0.5 g/L, 2,4-D 0.5 mg/L, kinetin 0.05 mg/L) and growing them under the conditions described above. Suspension cultures were established by inoculation of 300 mg of rapidly dividing, friable, white callus into 50 mL of Murashige and Skoog medium containing 0.5 mg/L naphthaleneacetic acid, 0.05 mg/L kinetin, 3% (w/v) Suc, and incubating them under the above conditions on a rotary shaker at 110 rpm. Cell suspensions were subcultured every 10 d for 3 months by decanting 60% of the medium and replacing it with fresh medium. Subculturing was subsequently carried out by pipetting 30 mL of the suspension into 170 mL of fresh medium in a 500-mL conical flask every 7 d. By this means, small cell aggregates and single cells were effectively selected. Using the conditions described, the cells turned green after 8 months and were then used for biochemical analysis.

Cell growth was determined by inoculating 0.5 mL of packed cell volume into a total volume of 50 mL of medium. Every 24 h, the cells were sedimented by centrifugation at 1700 rpm for 5 min and the packed cell volume was measured.

Cell Treatments

For the analysis of cell responses to stress, cells were treated on the 3rd d after subculturing (the calculated point at which cells entered exponential growth phase) with either the catalase inhibitor 3-amino-1,2,4-triazole (Sigma), or the inhibitor of γ-glutamylcysteine synthetase BSO (Sigma), or both. Inhibitors dissolved in sterile distilled water were added to a final concentration of 2 mM and 50 μM, respectively. To maximize reproducibility between experiments, the suspension cultures were treated with aminotriazole at the same point in the light/dark cycle, 2 h after the onset of the dark period. Any stimulation of GSH levels relative to T_0 values could thus be related to the effect of treatment, whereas limiting changes were associated with cell-growth effects.

At selected times after the addition of the inhibitor, 10-mL samples of the culture were removed, washed twice in fresh medium, and collected onto filter paper by vacuum aspiration. Cells were assayed for GSH, aconitase, catalase, and lipid peroxidation. All subsequent extractions were carried out at 4°C using ice-cold solutions. All measurements were

Table I. *The activities of aconitase and catalase and the levels of GSH in A. thaliana cells during the growth cycle*

Values are the mean ± SE of duplicate samples taken from two independent experiments.

Time	PCV	Reduced GSH	Aconitase	Catalase
d	*mL*	*nmol g^{-1} fresh weight*	*μmol NADP min^{-1} mg^{-1} protein*	*μmol O_2 min^{-1} mg^{-1} protein*
0	0.5	50 ± 0.1	0.65 ± 0.031	1.40 ± 0.062
2	2.5	55 ± 1.6	0.67 ± 0.024	1.40 ± 0.043
3	4	50 ± 0.25	0.65 ± 0.028	1.39 ± 0.055
4	7	50 ± 0.0	0.65 ± 0.060	1.40 ± 0.032
6	11	50 ± 1.2	0.63 ± 0.054	1.40 ± 0.012
9	13	55 ± 3.7	0.62 ± 0.041	1.38 ± 0.017

made in duplicate for each time point and each experiment was repeated three times. Preliminary results showed that most of the changes in metabolite pools or enzyme activities occurred during the first 4 h after treatment. Measurements were consequently taken throughout this period and at two later time points to assess cellular viability and structural damage.

GSH Assay

GSH concentrations in acid-soluble extracts of 200 mg fresh weight of tissue were assayed essentially as described by Smith (1985). Reduced GSH was determined as the difference between total GSH and GSSG.

Catalase Assay

Catalase was assayed essentially as described by Allen and Whatley (1978). Two hundred milligrams of tissue was ground in 1 mL of 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes, pH 7.6. One hundred microliters of the centrifuged extract was added to 900 μL of the grinding buffer and placed in an oxygen electrode. Hydrogen peroxide was added to a final concentration of 4 mM and the rate of oxygen evolution was measured. Catalase activity is expressed as μmol O_2 min^{-1} mg^{-1} protein.

Aconitase Assay

Samples of packed cells were ground using a mortar and pestle in 1 mL of a buffer containing 4 mM Cys, 5 mM EDTA, 0.1% (w/v) BSA, 10 mM Mops, adjusted to pH 7.4 with NaOH. Cell debris was precipitated by centrifugation at 12,000 rpm for 5 min, and the clear supernatant was used for the assay of aconitase essentially as described by Cooper and Beevers (1969) with the following modifications. The final assay mixture in 1 mL contained 100 μL of 0.148 M *cis*-aconitate (pH 6.5), 200 μL of extract, 500 μL of grinding buffer, 100 μL (0.2 units) of $NADP^+$-dependent isocitrate dehydrogenase, and the reaction was initiated by the addition of 100 μL of 2.5 mM $NADP^+$. Aconitase activity was calculated by recording the change in A_{340} and using the NADPH molar extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Lipid Peroxidation

Lipid peroxidation in 200-mg samples of cells was estimated essentially as described by Dhindsa and Matowe (1981) and is expressed as nmol malondialdehyde g^{-1} fresh weight. The concentration of malondialdehyde was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Protein Determination

Protein determinations were carried out using the method of Bradford (1976) with BSA as standard.

RESULTS

After 8 months of repeated subculturing, *A. thaliana* suspension cultures derived from stem explants showed both rapid cell division and fully undifferentiated morphology. Cells were assayed for their GSH content, lipid peroxidation levels, and the activities of aconitase and catalase. The results are summarized in Table I. The GSH content of the cells remained constant at around 50 nmol g^{-1} fresh weight except for a slight increase after subculturing and existed entirely in the reduced form. GSSG and lipid peroxidation (frequently used parameters indicative of oxidative stress [Dhindsa and Matowe, 1981; Dhindsa, 1991]) could not be detected throughout the growth cycle. The activities of both aconitase and catalase remained high and stable throughout the growth cycle.

Effect of Aminotriazole Treatment

Cells treated with 2 mM aminotriazole in the dark showed a rapid and continuous loss of catalase activity compared with untreated cells (Fig. 1A). Markers for the presence of hydrogen peroxide, aconitase (Fig. 1B), and lipid peroxidation

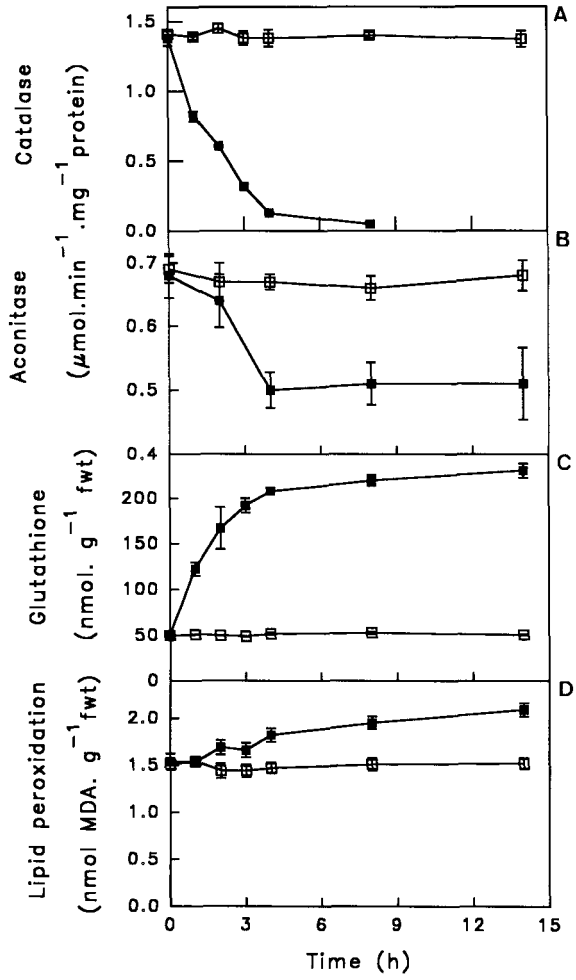

Figure 1. Effect of 2 mM aminotriazole on *A. thaliana* cell-suspension cultures. The activities of catalase (A) and aconitase (B) and the levels of GSH (C) and lipid peroxidation (D) were measured in extracts made from cells treated (■) or not (□) with 2 mM aminotriazole. Values are the mean of duplicate samples taken from three independent experiments; bars represent SE.

(Fig. 1D) were all affected but showed very different kinetics. Lipid peroxidation was observed to increase slightly 3 h after the addition of aminotriazole. Aconitase, however, showed an initial 25% decrease in activity during the 1st 4 h after addition of aminotriazole, but no additional decrease occurred, despite catalase activity being undetectable over a subsequent 10-h period. Cell viability, as measured by Evans blue exclusion, was not affected by this treatment and did not decline over a period of 72 h following the addition of aminotriazole (data not shown).

A 4-fold increase in the cellular content of GSH occurred over a period of 4 h after the addition of aminotriazole (Fig. 1C). Over a subsequent 10-h period, however, no further increase was observed. The level of GSSG was below the level of detection in aminotriazole-treated cultures for the 1st 3 h of treatment (less than 50 nmol g^{-1}), and was detected in only trace amounts at 4 h, with only a slight increase thereafter (68 ± 3.2 nmol g^{-1}).

Although the stimulation of the cellular GSH levels was marked, we also were interested in the possible causes for the arrest in its accumulation. It is known from previous studies demonstrating the accumulation of GSH in response to oxidative stress that the availability of sulfur can exert a limiting effect upon the final levels of GSH accumulated (Smith et al., 1985). To test if this is the case, 2 mM Na_2SO_4 was added to cells in the presence and absence of aminotriazole, which resulted in a slight increase in the levels of GSH (Table II). However, in the presence of aminotriazole, Na_2SO_4 addition did not stimulate GSH accumulation above the levels observed in the presence of aminotriazole alone, indicating that sulfur availability was not the cause of this effect.

Effect of GSH Depletion

An intriguing observation was that although the hydrogen peroxide-sensitive marker, aconitase, demonstrated the presence of hydrogen peroxide initially in aminotriazole-treated cells, subsequent decline in activity was not observed (Fig. 1B). In vitro it is known that the inhibition of aconitase by hydrogen peroxide is dependent upon both the length of the incubation period and the concentration of hydrogen peroxide used (Verniquet et al., 1991). Other oxidation-sensitive markers, GSSG (<50 nmol g^{-1}, T = 0 h; 68 ± 3.2 nmol g^{-1}, T = 14 h) and levels of lipid peroxidation (Fig. 1D), were not seen to be significantly elevated, demonstrating that the concentration of hydrogen peroxide present as a result of the inactivation of catalase was low and did not reach harmful levels. These data, taken together, suggested that during the period of incubation with aminotriazole, hydrogen peroxide initially leaked into the cytosol from the peroxisome, where its subsequent accumulation was limited by a cytosolic detoxification pathway.

We were interested in the possible role that the increase in reduced GSH levels played in preventing the accumulation of hydrogen peroxide, thereby protecting aconitase from oxidative inactivation. This was investigated by blocking GSH synthesis by use of the specific inhibitor of γ-glutamylcysteine synthetase, BSO. Since steady-state levels of GSH in these cultures were low, the inhibition of GSH synthesis was monitored by the ability of BSO to prevent aminotriazole-induced GSH accumulation. Incubation of suspension-cultured cells in the presence of BSO for 15 h prevented the accumulation of GSH upon addition of aminotriazole (data not shown). Viability of the cells subsequent to BSO addition was monitored over a 72-h period by their ability to exclude Evans blue and was found not to diminish (data not shown). Similarly, there was no effect upon aconitase activity (Fig. 2A) or levels of lipid peroxidation (Fig. 2C) during this period. Cells that had been depleted of GSH by BSO for 15 h and then treated with aminotriazole showed a rapid and continuous loss of aconitase and catalase activities (Fig. 2, A and B). Concomitantly, levels of lipid peroxidation rose more rapidly and more significantly (Fig. 2C) than in cells that had been treated with aminotriazole alone (Fig. 1D).

These events were followed 24 h later by a marked bleaching of the cells and a complete loss of viability as measured by Evans blue exclusion. Recombination experiments were carried out to demonstrate that this inactivation of aconitase occurs in vivo rather than during extraction. When an equal volume of an extract made from cells treated for 15 h with BSO then for 4 h with aminotriazole (aconitase activity; 0.09 ± 0.01 μmol NADP min^{-1} mg^{-1} protein) was mixed with an extract made from untreated cells (aconitase activity; 0.66 ± 0.028 μmol NADP min^{-1} mg^{-1} protein), an aconitase activity of 0.31 ± 0.022 μmol NADP min^{-1} mg^{-1} protein was measured. This is in good agreement with the predicted 43% lowering of activity, and thus much of the inhibition is likely to occur in vivo.

Table II. *The accumulation of GSH in the presence of extra added sulfate*

The effect of the addition of 2 mM sodium sulfate to cell cultures grown in the presence or absence of 2 mM aminotriazole on GSH levels was measured. The control samples received no additions. Values are the mean ± SE of duplicate samples taken from two independent experiments.

Treatment	GSH
	nmol g^{-1}
Control	50 ± 0.15
2 mM Aminotriazole	214 ± 8.7
2 mM Na_2SO_4	55 ± 0.2
2 mM Na_2SO_4 + 2 mM aminotriazole	225 ± 8.9

Recovery of GSH Levels after Removal of BSO and Susceptibility of These Cells to Oxidative Injury

The recovery of GSH levels after removal of BSO-containing medium and its replacement with fresh medium was followed over a 20-h period. These data are summarized in Table III. Resynthesis of GSH in these cultures was rather slow, probably reflecting slow dissociation of enzyme-inhibitor complexes and metabolism of residual BSO. GSH was undetectable at 5 h; however, after 10 h, levels of GSH exceeded those seen in untreated cells, and after 20 h, a 3-fold elevation of the level was observed.

We examined the sensitivity of aconitase activity and lipid peroxide levels to aminotriazole treatment during this recov-

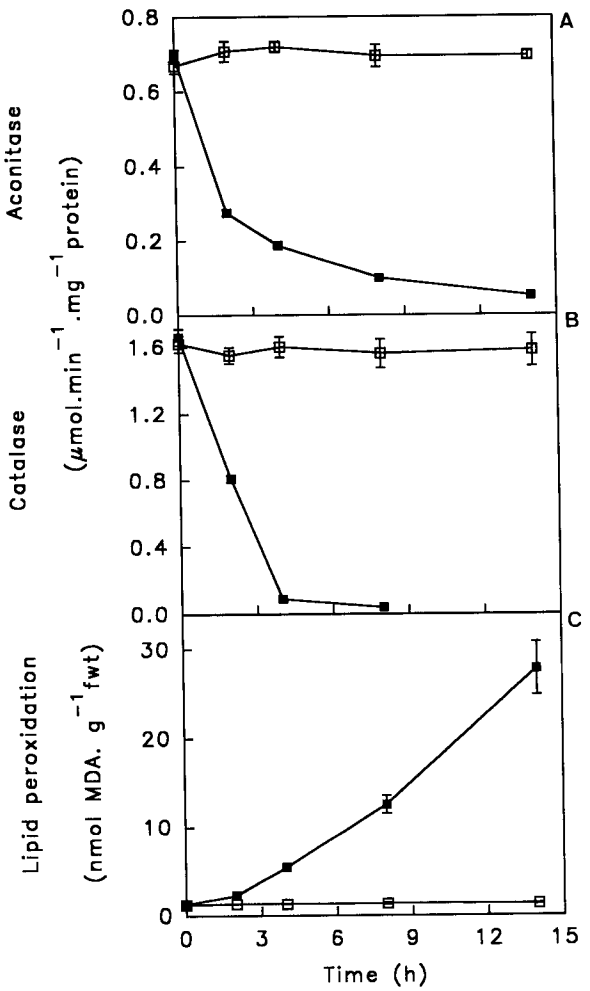

Figure 2. The effects of aminotriazole treatment on cells treated with BSO for 15 h. Measurements of aconitase (A), catalase (B), and the products of lipid peroxidation (C) were made at the time point indicated in the presence (■) or absence (□) of 2 mM aminotriazole in extracts made from cells pretreated with 50 μM BSO for 15 h. Values are the means of duplicate samples taken from two (A and C) and three (B) independent experiments; bars represent SE.

ery period. Cells were treated with 2 mM aminotriazole for 1 h and then measurements were made as described. Initially, after growth of cells in fresh medium for 0 or 5 h, followed by the addition of aminotriazole, the inhibition of catalase caused a sharp drop in aconitase activity relative to the activity at T_0 (Table IV) and a significant increase in levels of lipid peroxidation (Table IV). However, resynthesis of GSH after 10 h in fresh medium to levels 1.6 times the initial level (Table IV) was seen to be correlated with increased protection of aconitase. Furthermore, after 20 h of growth in fresh medium followed by the addition of 2 mM aminotriazole for 1 h, where a markedly elevated level of reduced GSH was measured (3.6 times the initial value), complete protection of both markers was observed. Analysis of the ratio of reduced GSH/GSSG in response to aminotriazole during this recovery period showed that accumulation of GSSG above the level of detection was only measurable at 10 h, whereas at 20 h in the presence of aminotriazole, all GSH detected was in the reduced form.

DISCUSSION

Our interest is in the mechanisms underlying the increase in GSH levels that have been observed in response to the presence of hydrogen peroxide in plants (Smith et al., 1984, 1985; Smith, 1985). A primary aim of the present study was to establish a system in which hydrogen peroxide could be generated and detected intracellularly.

Measurement of aconitase activity in *Arabidopsis* suspension-cell cultures treated with the catalase inhibitor aminotriazole demonstrated a measurable yet small initial decrease. Given the extreme sensitivity of aconitase to oxidation, its use as a marker in this system allows us to infer that, in response to aminotriazole treatment, hydrogen peroxide was present in the cytosol, albeit transiently and in low concentration. The subsequent arrest of this inhibition suggests that the hydrogen peroxide present initially was removed because, even if it were leaking into the cytosol in low amounts, its accumulation over time would nevertheless lead to a gradual increase in inhibition of aconitase. Similarly, our failure to detect increases in the levels of the products of lipid peroxidation or an accumulation of GSSG, despite decreasing catalase activity, strongly supports the proposition that a catalase-independent cytosolic detoxification pathway was

Table III. *The recovery of GSH levels in suspension cultures after treatment with BSO*

Cells were treated with 50 μM BSO for 15 h and the medium was removed and replaced with fresh medium. The recovery of GSH levels was monitored over a 20-h period at 5, 10, and 20 h after the removal of BSO. Aconitase activity and the levels of lipid peroxidation were measured in each sample to determine the level of oxidative injury. Values are the mean of duplicate samples taken from three (reduced GSH and lipid peroxidation) and four (aconitase) independent experiments.

Treatment	Reduced GSH	Aconitase	Lipid Peroxidation
	nmol g^{-1}	*μmol NADP* min^{-1} mg^{-1}	*nmol malondialdehyde* g^{-1}
t = 0	55 ± 4.2	0.66 ± 0.028	0.94 ± 0.12
15 h BSO	<50	0.65 ± 0.029	1.20 ± 0.04
15 h BSO, 5 h fresh medium	<50	0.67 ± 0.035	1.25 ± 0.09
15 h BSO, 10 h fresh medium	85 ± 6.7	0.66 ± 0.065	1.21 ± 0.03
15 h BSO, 20 h fresh medium	175 ± 12.2	0.69 ± 0.053	1.19 ± 0.06

Table IV. *Effects of aminotriazole on markers for oxidative injury and GSH levels in cells recovering from BSO treatment*

Cells were treated with 50 μM BSO for 15 h and the medium was removed and replaced with fresh medium. At 0, 5, 10, or 20 h following removal of BSO, aminotriazole was added to a final concentration of 2 mM. The level of GSH and lipid peroxidation and the activity of aconitase were measured 1 h after the addition of aminotriazole. Values are the mean ± SE of duplicate samples taken from three independent experiments.

Treatment	Reduced GSH	GSSG	Aconitase	Lipid Peroxidation
	nmol g^{-1}	*nmol g^{-1}*	*μmol NADP min^{-1} mg^{-1} protein*	*nmol malondialdehyde g^{-1}*
t = 0	55	<50	0.65 ± 0.044	0.95 ± 0.16
15 h BSO, 1 h aminotriazole	<50	<50	0.22 ± 0.03	6 ± 0.14
15 h BSO, 5 h fresh medium, 1 h aminotriazole	<50	<50	0.25 ± 0.10	16 ± 0.28
15 h BSO, 10 h fresh medium, 1 h aminotriazole	120 ± 2.7	66.58	0.56 ± 0.04	2.1 ± 0.07
15 BSO, 20 h fresh medium, 1 h aminotriazole	280 ± 12.1	<50	0.68 ± 0.02	1.18 ± 0.12

responsible for removing hydrogen peroxide before it accumulated to harmful levels.

Our data also suggest that the cytosolic antioxidant capacity is sufficient to maintain cell viability in the absence of catalase, and we show that under such conditions, a 4-fold increase in the level of reduced GSH can be measured. The fact that a significant increase in the level of reduced GSH was observed despite only a transient accumulation of hydrogen peroxide suggests that the mechanism of hydrogen peroxide-induced GSH synthesis is much more complex than was previously proposed.

GSH synthesis is known to be regulated both by the supply of substrates, notably Cys, and also by feedback inhibition of γ-glutamylcysteine synthetase by reduced GSH (Rennenberg, 1982; Hell and Bergmann, 1990). Current models describing the stimulation of GSH levels in response to an oxidative stimulus propose that accumulation results from an overloading of the system with reactive oxygen species so that the capacity for GSH reductase to maintain GSH in the reduced form is exceeded and GSSG accumulates. As the level of reduced GSH declines, γ-glutamylcysteine synthetase is released from feedback inhibition and synthesis is stimulated (Richman and Meister, 1975; Alscher, 1989). The accumulation of GSSG will reflect an inadequate GSH reductase activity or a too-low NADPH:NADP ratio and a sufficiently high hydrogen peroxide concentration. Complications to this model arise through demonstrations that large variations in the level of GSH accumulated depend upon the plant species, leaf age, the basal level of GSH, and the ratio of reduced GSH:GSSG accumulated (Smith, 1985). Other studies have shown that in response to gaseous pollutants in some (Guri, 1983; Madamanchi and Alscher, 1991) but not all (Sen Gupta et al., 1991) cases, the proportion in GSSG remains small while total GSH levels rise.

The mildness of the oxidative stimulus in our system distinguishes our approach from previous studies, and we feel that differences between observations made in our system and those of Smith and colleagues (Smith et al., 1984, 1985; Smith, 1985) simply reflect differences in the severity of the stress applied. In the latter, structural damage to chloroplasts and subsequent cell death was attributed to the accumulation of hydrogen peroxide arising from photorespiration in the absence of catalase activity. In our system, in which photorespiration did not occur, structural damage through oxidative stress was not observed. Neither was any loss in cell viability observed, unless GSH levels depleted. Although our observations indicate a role for hydrogen peroxide in the stimulation of GSH levels, we have as yet no idea of the nature of the signals linking these two phenomena.

An important goal of this work was to determine the physiological role of the observed increase in the levels of reduced GSH, since the outcome of a potentially harmful oxidative event is determined by the cellular antioxidant capacity. Our data and those obtained from other experimental systems suggest that an important contributory element is that plants can respond to an oxidative stimulus with the synthesis of more antioxidant molecules. The outcome would then depend upon the speed of synthesis of the antioxidants as well as their subsequent ability to play a continuous role if the stimulus were both sustained and severe. To investigate the physiological significance of the increase in reduced GSH levels, we treated cells with BSO for 15 h and subsequently examined the activity of oxidation-sensitive markers. In the absence of GSH synthesis, there was no evidence for an increase in oxidative events. In contrast, addition of aminotriazole to BSO-treated cells resulted in massive oxidative damage (Fig. 2, A and C) and subsequent cell death. Clearly, in the absence of a sufficiently large reduced GSH pool, which in our system is generated in response to mild oxidative stress, hydrogen peroxide accumulates in the cytosol and causes oxidative damage. This approach allows a clear discrimination to be made between the consequences of GSH depletion and those arising through stress in the absence of GSH, since the means of depletion is not a stress in itself (Table III), is highly specific, and, as we and others (Arrick et al., 1982) have shown, is otherwise nontoxic to the cell.

Further demonstrations of the importance of reduced GSH

as an antioxidant were provided in subsequent experiments in which cells recovering from BSO were challenged with aminotriazole (Table IV). After 10 h of growth in fresh medium followed by exposure to aminotriazole for 1 h, when a 2-fold increase in GSH levels was measured, partial protection of aconitase, some accumulation of GSSG, and accumulation of the products of lipid peroxidation were observed. However, after 20 h, where GSH levels were markedly stimulated in response to aminotriazole challenge, these markers for oxidative injury no longer responded, indicating full recovery of cytosolic protective mechanisms. Thus, during the period of recovery there was a strong correlation between the cellular concentration of GSH and the capacity of the cells to resist oxidative damage consequent upon the presence of hydrogen peroxide. Taken together, these data demonstrate that the ability of cells to withstand a potentially damaging oxidative stimulus is dependent at least in part upon the capacity for de novo GSH synthesis and that the frequently observed increase in the levels of GSH in response to an oxidative stimulus can play a crucial role in cellular protection. This approach complements similar studies made in animal systems that demonstrated the utility of BSO as an analytical tool for the study of GSH and the crucial role it plays in cellular defense as an antioxidant during oxidative stress (Griffith and Meister, 1979; Arrick et al., 1982).

A distinct advantage of our system is that growth conditions remain stable and the potential for oxidative stress can be manipulated at precise time points. A further advantage is that we were able to determine the importance of events underlying the balance between pro- and antioxidants early in the onset of an oxidative stress. It appears clear that the outcome of this response in plants depends upon a rapid activation of GSH synthesis. In vivo, the measurable effects of an oxidative stimulus will be the result of antagonistic pro- and antioxidants coupled with the cell's capacity to respond with the synthesis of more antioxidants. This raises the intriguing possibility that prior exposure to a mild stress could confer tolerance to a subsequently severe stress.

This study provides further evidence for the role of signals generated through the presence of hydrogen peroxide in the regulation of GSH synthesis. A major priority remains to determine the nature of these signals and to understand how the GSH biosynthetic pathway responds to their presence.

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